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RECOMBINANT ADENOVIRAL VECTOR

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RECOMBINANT ADENOVIRAL VECTOR

BACKGROUND OF THE INVENTION

Throughout this application, various publications are referred to by citations within parentheses. The disclosures of these publications are incorporated by reference into the present disclosure.

Production of recombinant adenoviruses useful for gene therapy requires the use of a cell line capable of supplying in *trans* the gene products of the viral E1 region which are deleted in these recombinant viruses. At present the only useful cell line available is the 293 cell line originally described by Graham et al. in 1977 (Graham, F.L. et al., J. Gen. Virol. 36:59-74 (1977)). 293 cells contain approximately the left hand 12% (4.3 kb) of the adenovirus type 5 genome (Aiello, L. et al., Virology 94:460-469 (1979) and Spector, D.J., Virology 130:533-538 (1983)).

Adenoviral vectors currently being tested for gene therapy applications typically are deleted for Ad2 or Ad5 DNA extending from approximately 400 base pairs from the 5′ end of the viral genome to approximately 3.3 kb from the 5′ end, for a total E1 deletion of 2.9 kb. Therefore, there exists a limited region of homology of approximately 1 kb between the DNA sequence of the recombinant virus and the Ad5 DNA within the cell line. This homology defines a region of potential recombination between the viral and cellular adenovirus sequences. Such a recombination results in a phenotypically wild-type virus bearing the Ad5 E1 region from the 293 cells. This recombination event presumably accounts for the frequent detection of wild-type adenovirus in preparations of recombinant virus and has been directly demonstrated to be the cause of wild-type contamination of the Ad2 based recombinant virus Ad2/CFTR-1 (Rich, D.P., et al. Hum. Gene Therapy, Vol. 4:460-476 (1993)).

Due to the high degree of sequence homology within the type C adenovirus subgroup such recombination is likely to occur if the vector is based on any group C adenovirus (types 1, 2, 5, 6).

In small scale production of recombinant adenoviruses, generation of contaminating wild-type virus can be managed by a screening process which discards those preparations of virus found to be contaminated. As the scale of virus production grows to meet expected demand for genetic therapeutics, the likelihood of any single lot being contaminated with a wild-type virus also will rise as well as the difficulty in providing non-contaminated recombinant preparations.

Thus, because of the increasing use of large scale recombinant production of viruses, and the likelihood of wild-type contamination in such preparations, a need exists for a vector not subject to recombination to produce wild-type virus in these preparations. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides a recombinant adenovirus expression vector characterized by the inability to express adenoviral protein IX DNA and having the ability to express a foreign gene. Transformed host cells and a method of producing recombinant proteins and gene therapy also are included within the scope of this invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a recombinant adenoviral vector of this invention. This construct was assembled as shown in Figure 1. The resultant virus bears a 5′ deletion of

adenoviral sequences extending from nucleotide 356 to 4020 and eliminates the E1a and E1b genes as well as the entire protein IX coding sequence, leaving the polyadenylation site shared by the E1b and pIX genes intact for use in terminating transcription of any desired gene.

Figure 2 shows the amino acid sequence of $p110^{RB}$.

Figure 3 shows a DNA sequence encoding a retinoblastoma tumor suppressor protein.

Figure 4 shows schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have ahd the E1 region of nucleotides 360-3325 replaced with a 1.4 kb full length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus but lacks the 1.4 kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9 kb Xba I deletion within adenovirus type 5 region E3.

Figure 5 shows p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A.) Saos-2 (osteosarcoma) cells were infected at the indicated multiplicities of infection (MOI) with either the A/M/53 or A/C/53 purified virus and harvested 24 hours later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentration of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. "O" under the A/C/53 heading indicates a mock infection, containing untreated Saos-2 lysate. B.) Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated MOI and analyzed as

in part A.) The arrow indicates the position of the p53 protein.

Figure 6 shows p53 dependent Saos-2 morphology change. Subconfluent (1 x 10^5 cells/10 cm plate) Saos-2 cells were either uninfected (A), infected at an MOI = 50 with (B) the control A/M virus or (C) the A/C/53 virus. The cells were photographed 72 hours post-infection.

Figure 7 shows p53 dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M (-x-x-), or the p53 expressing A/M/N/53 (-Δ-Δ-), or A/C/N/53 (-O-O-) virus at increasing MOI as indicated. The tumor type and p53 status is noted for each cell line (wt = wild type, null = no protein expressed, mut = mutant protein expressed). DNA synthesis was measured 72 hours post-infection as described in Methods. Results are from triplicate measurements at each dose (mean+/- SD), and are plotted as % of media control versus MOI. * H69 cells were only tested with A/M and A/M/N/53 virus.

Figure 8 shows tumorigenicity of p53 infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at MOI = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Methods) twice per week for 8 weeks. Results are plotted as tumor size versus days post tumor cell implantation for both control A/M (-x-x-) and A/M/N/53 (-Δ-Δ-) treated cells. Error bars represent the mean tumor size =/- SEM for each group of 4 animals at each time point.

Figure 9 is expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25-50 mm_3. Mice were randomized and injected peritumorally with 2 x 10_9 pfu of either control A/C/ß-gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and polyA RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min., 55°C 1.5 min., 72°C 2 min., and a 10 min., 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5′ Tripartite Leader cDNA (5′ - CGCCACCGAGGGACCTGAGCGAGTC-3′) and a 3′ p53 primer (5′ - TTCTGGGAAGGGACAGAAGA-3′). Lanes 1,2,4, and 5 are p53 treated samples excised at day 2 or 7 as indicated. Lanes 3 and 6 are from ß-gal treated tumors. Lanes 7,8, and 9 are replicates of lanes 4,5, and 6 respectively, amplified with actin primers to verify equal loading. Lane 10 is a positive control using a tripartite/p53 containing plasmid.

Figure 10 shows *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (---), control A/M adenovirus (-x-x-), or A/M/N/53 (-Δ-Δ), (both virus 2 x 10^9 pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Methods. A) Tumor size is plotted for each virus versus time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size +/- SEM for each group of 5 animals. Arrows indicate days virus injections. B) Mice were monitored for survival and the fraction of mice surviving per group versus time post

inoculation of buffer alone (----), control A/M (··· ··· ···) or A/M/N/53 (----) virus treated H69 cells is plotted.

DETAILED DESCRIPTION OF THE INVENTION

To reduce the frequency of contamination with wild-type adenovirus, it is desirable to improve either the virus or the cell line to reduce the probability of recombination. For example, an adenovirus from a group with low homology to the group C viruses could be used to engineer recombinant viruses with little propensity for recombination with the Ad5 sequences in 293 cells. However, an alternative, easier means of reducing the recombination between viral and cellular sequences is to increase the size of the deletion in the recombinant virus and thereby reduce the extent of shared sequence between it and the Ad5 genes in the 293 cells.

Deletions which extend past 3.5 kb from the 5′ end of the adenoviral genome affect the gene for adenoviral protein IX and have not been considered desirable in adenoviral vectors (see below).

The protein IX gene of the adenoviruses encodes a minor component of the outer adenoviral capsid which stabilizes the group-of-nine hexons which compose the majority of the viral capsid (Stewart, P.L. et al., <u>EMBO Journal</u> 12:2589-2599 (1993)). Based upon study of adenovirus deletion mutants, protein IX initially was thought to be a non-essential component of the adenovirus, although its absence was associated with greater heat lability than observed with wild-type virus (Colby, W.W. and Shenk, T., <u>J. Virology</u> 39:977-980 (1981)). More recently it was discovered that protein IX was essential for packaging full length viral DNA into capsids and that in the absence of protein IX, only genomes at least 1 kb smaller than wild-type could be propagated as recombinant

viruses (Ghosh-Choudhury, G. et al., EMBO Journal 6:1733-1739 (1987)). Given this packaging limitation, pIX deletions have not been deliberately considered in the design of adenoviral vectors.

This invention claims the use of recombinant adenoviruses bearing deletions of the protein IX gene as a means of reducing the risk of wild-type adenovirus contamination in virus preparations for use in gene therapy applications. These deletions can remove an additional 500 to 700 base pairs of DNA sequence that is present in conventional E1 deleted viruses (smaller, less desirable, deletions of portions of the PIX gene are possible and are included within the scope of this invention), and is available for recombination with the Ad5 sequences integrated in 293 cells. Recombinant adenoviruses based on any group C virus, serotype 1, 2, 5 and 6, are included in this invention. Also encompassed by this invention is a hybrid Ad2/Ad5 based recombinant virus expressing the human p53 CDNA from the adenovirus type 2 major late promoter. This construct was assembled as shown in Figure 1. The resultant virus bears a 5′ deletion of adenoviral sequences extending from about nucleotide 357 to 4020 and eliminates the E1a and E1b genes as well as the entire protein IX coding sequence, leaving the polyadenylation site shared by the E1b and pIX genes intact for use in terminating transcription of any desired gene. Alternatively the deletion can be extended an additional 30 to 40 base pairs without affecting the adjacent gene for protein IVa2, although in that case an exogenous polyadenylation signal is provided to terminate transcription of genes inserted into the recombinant virus. The initial virus constructed with this deletion is easily propagated in 293 cells with no evidence of wild-type viral contamination and directs robust p53 expression from the transcriptional unit inserted at the site of the deletion.

The insert capacity of recombinant viruses bearing the pIX deletion described above is approximately 2.6 kb. This is sufficient for many genes including the p53 cDNA. Insert capacity can be increased by introducing other deletions into the adenoviral backbone, for example, deletions within early regions 3 or 4 (for review see: Graham, F.L. and Prevec, L. Methods in Molecular Biology Volume 7: Gene Transfer and Expression Protocols, 109-128 (1991)). For example, the use of an adenoviral backbone containing a 1.9 kb deletion of non-essential sequence within early region 3. With this additional deletion, the insert capacity of the vector is increased to approximately 4.5 kb, large enough for many larger cDNAs, including that of the retinoblastoma tumor suppressor gene.

A recombinant adenovirus expression vector characterized by the inability to express adenoviral protein IX DNA and having the ability to express a foreign gene is provided by this invention. These vectors are useful for the safe recombinant production of diagnostic and therapeutic polypeptides and proteins, and more importantly, for the introduction of genes for gene therapy. It can be used with any expression cassette. An "expression cassette" means a DNA molecule having a transcription promoter, a foreign gene, and in some embodiments defined below, a polyadentlyation signal. As used herein, the term "foreign gene" is intended to mean a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in wild-type adenovirus. The foreign gene is a DNA molecule up to 2.6 kilobases. "Expression vector" means a vector that results in the expression of inserted DNA sequences when propagated in a suitable host cell, i.e., the protein or polypeptide coded for by the DNA is sythesised by the host's system. The recombinant adenovirus expression vector can contain all or part of the gene encoding adenovirus protein IX, provided that biologically active protein IX is not

produced. An example of this vector is an expression vector having the restriction enzyme map of Figure 1.

Also provided by this invention is a recombinant adenovirus expression vector, as described above, having less extensive deletions of the protein IX gene sequence extending from 3500 bp from the 5′ viral termini to approximately 4000 bp, in one embodiment. In a separate embodiment, the recombinant adenovirus expression vector can have a further deletion of a non-essential DNA sequence in adenovirus early region 3 and/or 4 and/or deletion of the DNA sequences designated adenovirus E1a and E1b. In this embodiment, foreign gene is a DNA molecule of a size up to 4.5 kilobases.

A further embodiment has a further deletion of up to forty nucleotides positioned 3′ to the E1a and E1b deletion and pIX and a foreign DNA molecule encoding a polyadenylation signal inserted into the recombinant vector in a position relative to the foreign gene to regulate the expression of the foreign gene.

For the purposes of this invention, the recombinant adenovirus expression vector can be derived from wild-type Group adenovirus, serotype 1, 2, 5 or 6.

In one embodiment, the recombinant adenovirus expression vector has a foreign gene coding for a tumor suppressor gene, e.g., a retinoblastoma tumor suppressor protein or a biologically active fragment thereof. The complete RB cDNA nucleotide sequences and predicted amino acid sequences of the resulting RB protein (designated $p110^{RB}$) are shown in Lee et al., <u>Science</u> Vol.235:1394-1399 (1987), incorporated herein by reference. Also useful to express retinoblastoma tumor suppressor protein is a DNA molecule encoding the amino acid sequence shown in Figure 2 or having the DNA sequence shown in Figure 3. A

truncated version of p110[RB], called p56[RB] also is useful. For the sequence of p56[RB], see Huang, et al. Nature Vol. 350:160-162 (1991), incorporated herein by reference.

A recombinant adenovirus expression vector having a foreign gene coding for p53 protein or an active fragment thereof is provided by this invention. The coding sequence of the p53 gene is set forth below in Table I.

TABLE 1

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                                                           50
V*SHR PGSR* LLGSG DTLRS GWERA FHDGD TLPWI GSQTA FRVTA MEEPQ
                                                          100
SDPSV EPPLS QETFS DLWKL LPENN VLSPL PSQAM DDLML SPDDI EQWFT
                                                          150
EDPGP DEAPR MPEAA PPVAP APAAP TPAAP APAPS WPLSS SVPSQ KTYQG
                                                          200
SYGFR LGFLH SGTAK SVTCT YSPAL NKMFC QLAKT CPVQL WVDST PPPGT
                                                          250
RVRAM AIYKQ SQHMT EVVRR CPHHE RCSDS DGLAP PQHLI RVEGN LRVEY
                                                          300
LDDRN TFRHS VVVPY EPPEV GSDCT TIHYN YMCNS SCMGG MNRRP ILTII
                                                          350
TLEDS SGNLL GRNSF EVRVC ACPGR DRRTE EENLR KKGEP HHELP PGSTK
                                                          400
RALPN NTSSS PQPKK KPLDG EYFTL QIRGR ERFEM FRELN EALEL KDAQA

GKEPG GSRAH SSHLK SKKGQ STSRH KKLMF KTEGP DSD*
* = Stop codon
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Further provided by this invention is a transformed eucaryotic host cell having inserted a recombinant adenovirus expression vector described above. Any cell line expressing E1a and E1b or E1a, E1b and pIX is a suitable host for this vector. In one embodiment, the eucaryotic host cell is the 293 cell line available from

the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20231.

A method of producing a polypeptide or protein by growing the transformed eucaryotic host cell described above under conditions favoring transcription and translation of the foreign gene and isolating the polypeptide or protein so produced is also within the scope of this invention as well as a purified polypeptide or protein produced by this method. As used herein, purified or isolated mean substantially free of native proteins or nucleic acids normally associated with the protein or polypeptide in the native or host cell environment.

The vectors of this invention are particularly suited for gene therapy and methods of gene therapy utilizing these vectors is within the scope of this invention. The vector is purified and then an effective amount is administered *in vivo* or *ex vivo* into the subject. "Subject" means any animal, mammal, murine or human patient. The foreign gene can code for a tumor suppressor gene or other anti-cancer protein to treat or reduce hyperproliferative cells in a subject. Pathologic hyperproliferative cells are characteristic of the following disease states, thyroid hyperplasia - Grave's Disease, psoriasis, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias and lymphomas. Examples of non-pathologic hyperproliferative cells are found, for instance, in mammary ductal epithelial cells during development of lactation and also in cells associated with wound repair. Pathologic hyperproliferative cells characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a change in the surface properties of the cell and a further breakdown in intercellular communication. These

changes include stimulation to divide and the ability to secrete proteolytic enzymes.

Moreover, the present invention relates to a method for depleting a suitable sample of pathologic mammalian hyperproliferative cells contaminating hematopoietic precursors during bone marrow reconstitution via the introduction of a wild type tumor suppressor gene into the cell preparation using the vector of this invention (whether derived from autologous peripheral blood or bone marrow). As used herein, a "suitable sample" is defined as a heterogeneous cell preparation obtained from a patient, e.g., a mixed population of cells containing both phenotypically normal and pathogenic cells. "Administer" includes, but it not limited to introducing into the cell or subject intravenously, by direct injection into the tumor, by aerosol administration to the lung or topically,

A method of gene therapy by administering to a subject or a cell, an effective amount of a vector described above is provided by this invention. Also within the scope of this invention is a method of ameliorating a pathology characterized by hyperprolierative cells or genetic defect in a subject by administering to the subject an effective amount of a vector described above containing a foreign gene encoding a gene product having the ability to ameliorate the pathology, under suitable conditions. As used herein, the term "genetic defect" means any disease or abnormality that results from inherited factors, such as sickle cell anemia or Tay-Sachs disease.

The use of the adenoviral vector of this invention to prepare medicaments for the treatment of a disease or for therapy is further provided by this invention.

The following examples are intended to illustrate, not limit the scope of this invention.

EXPERIMENT NO. I

Plasmid pAd/MLP/p53/E1b- was used as the starting material for these manipulations. This plasmid is based on the pBR322 derivative pML2 (pBR322 deleted for base pairs 1140 to 2490) and contains adenovirus type 5 sequences extending from base pair 1 to base pair 5788 except that it is deleted for adenovirus type 5 base pairs 357 to 3327. At the site of the Ad5 357/3327 deletion a transcriptional unit is inserted which is comprised of the adenovirus type 2 major late promoter, the adenovirus type 2 tripartite leader cDNA and the human p53 cDNA. It is a typical E1 replacement vector deleted for the Ad5 E1a and E1b genes but containing the Ad5 protein IX gene (for review of Adenovirus vectors see: Graham, F.L. and Prevec, L. <u>In</u>: Vaccines: New Approaches to Immunological Problems. R.W. Ellis (ed), Butterworth-Heinemann, Boston. pp 363-390 (1992)). Ad2 DNA was obtained from Gibco BRL. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. *E. coli* DH5α competent cells were purchased from Gibco BRL and 293 cells were obtained from the American Type Culture Collection (ATCC). Prep-A-Gene DNA purification resin was obtained from BioRad. LB broth bacterial growth medium was obtained from Difco. Qiagen DNA purification columns were obtained from Qiagen, Inc. Ad5 dl327 was obtained from R.J. Schneider, NYU. The MBS DNA transfection kit was purchased from Stratagene.

One (1) μg pAd/MLP/p53/E1b- was digested with 20 units each of restriction enzymes Ecl 136II and NgoMI according to the manufacturer's recommendations. Five (5) μg Ad2 DNA was digested with 20 units each of restriction endonucleases DraI and NgoMI according to the manufacturer's recommendations. The restriction digestions

were loaded into separate lanes of a 0.8% agarose gel and electrophoresed at 100 volts for 2 hours. The 4268 bp restriction fragment from the pAd/MLP/p53/E1b- sample and the 6437 bp fragment from the Ad2 sample were isolated from the gel using Prep-A-Gene DNA extraction resin according to the manufacturer's specifications. The restriction fragments were mixed and treated with T4 DNA ligase in a total volume of 50 μl at 16°C for 16 hours according to the manufacturer's recommendations. Following ligation 5 μl of the reaction was used to transform *E. coli* DH5α cells to ampicillin resistance following the manufacturer's procedure. Six bacterial colonies resulting from this procedure were used to inoculate separate 2 ml cultures of LB growth medium and incubated overnight at 37°C with shaking. DNA was prepared from each bacterial culture using standard procedures (Sambrook, J. et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (1989)). One fourth of the plasmid DNA from each isolate was digested with 20 units of restriction endonuclease XhoI to screen for the correct recombinant containing XhoI restriction fragments of 3627, 3167, 2466 and 1445 base pairs. Five of six screened isolates contained the correct plasmid. One of these was then used to inoculate a 1 liter culture of LB medium for isolation of large quantities of plasmid DNA. Following overnight incubation plasmid DNA was isolated from the 1 liter culture using Qiagen DNA purification columns according to the manufacturer's recommendations. The resulting plasmid was designated pAd/MLP/p53/pIX-.

To construct a recombinant adenovirus, 10 μg pAd/MLP/p53/pIX- were treated with 40 units of restriction endonuclease EcoRI to linearize the plasmid. Adenovirus type 5 dl327 DNA (Thimmappaya, B. et al. Cell 31:543-551 (1982)) was digested with restriction endonuclease ClaI and the large fragment (approximately 33 kilobase pairs) was purified by sucrose gradient centrifugation. Ten (10) μg

of EcoRI treated pAd/MLP/p53/E1b- and 2.5 μg of ClaI treated Ad5 dl327 were mixed and used to transfect approximately 10^6 293 cells using the MBS mammalian transfection kit as recommended by the supplier. Eight (8) days following the transfection the 293 cells were split 1 to 3 into fresh media and two days following this adenovirus induced cytopathic effect became evident on the transfected cells. At 13 days post-transfection DNA was prepared from the infected cells using standard procedures (Graham, F.L. and Prevec, L. In: Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Humana Press, Clifton, N.J. (1991)) and analyzed by restriction digestion with restriction endonuclease XhoI. Virus directed expression of p53 was verified following infection of SaoS2 osteosarcoma cells with viral lysate and immunoblotting with an anti-p53 monoclonal antibody designated 1801 (Novocasta Lab. Ltd., U.K.).

EXPERIMENT NO. II

MATERIALS AND METHODS

Cell Lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

Construction of Recombinant Adenoviruses

To construct the Ad5/p53 viruses, a 1.4 kb HindIII-SmaI fragment containing the full length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP71 (Promega) using standard cloning procedures (Sambrook *et al*, 1989). The p53 insert was recovered from this vector following digestion with XhoI-BgIII and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider) which contain the Ad5 s' inverted terminal repeat and viral packaging signals and the Ela enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3325-5525 bp in a pML2 background. These new constructs replace the E1 region (bp 360-3325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Figure 4). The p53 inserts use the remaining downstream Elb polyadenylation site. Additional MLP and CMV driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705 nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second control consisted of a recombinant adenovirus encoding the beta-galactosidase gene under the control of the CMV promoter (A/C/ß-gal). The plasmids were linearized with either Nru I or Eco RI and co-transfected with the large fragment of a Cla I digested Ad 5 dI309 or dI327 mutants (Jones and Shenk, 1979) using a Ca/PO_4 transfection kit (Stratagene). Viral plaques were isolated and recombinants identified by both restriction digest analysis and PCR using recombinant specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by

limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973, Graham and Prevec, 1991).

p53 Protein Detection

Saos-2 or Hep 3B cells (5 x 10^5) were infected with the indicated recombinant adenoviruses for a period of 24 hours at increasing multiplicities of infection (MOI) of plaque forming units of virus/cell. Cells were then washed once with PBS and harvested in lysis buffer (50mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP40, 50mM NaF, 5mM EDTA, 10ug/ml aprotinin, 10 ug/ml leupeptin, and 1mM PMSF). Cellular proteins (approximately 30 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with α-p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA Synthesis Rate

Cells (5 x 10^3/well) were plated in 96-well titer plates (Costar) and allowed to attach overnight (37ºC, 7% CO_2). Cells were then infected for 24 hours with purified recombinant virus particles at MOIs ranging from 0.3 to 100 as indicated. Media were changed 24 hours after infection, and incubation was continued for a total of 72 hours. ^{3}H-thymidine (Amersham, 1μCi/well) was added 18 hours prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. ^{3}H-thymidine incorporation was expressed as the mean % (+/- SD) of media control and plotted versus the MOI.

Tumorigenicity in Nude Mice

Approximately 2.4 x 10^8 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53 or A/M purified virus at an MOI of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53 treated cells, while the contralateral flank was injected with the control A/M treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer treated cells served as additional controls. Tumor dimensions (length, width and height) and body weights were then measured twice per week over an 8 week period. Tumor volumes were estimated for each animal assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intra-tumoral RNA Analysis

BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1 x 10^7 H69 small cell lung carcinoma (SCLC) cells in their right flanks. Tumors were allowed to progress for 32 days until they were approximately 25-50 mm^3. Mice received peritumoral injections of either A/C/53 or A/C/ß-gal recombinant adenovirus (2 x 10^9 plaque forming units (pfu)) into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). PolyA RNA was isolated using the PolyATract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for RT-PCR determination of recombinant p53 mRNA expression (Wang *et al,* 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and

the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 Gene Therapy of Established Tumors in Nude Mice

Approximately 1 x 10^7 H69 (SCLC) tumor cells in 200μl volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size (N=5/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2 x 10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of Recombinant p53-Adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus Type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells which supply Ad 5 E1 gene products in *trans* (Graham *et al*, 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild

type adenovirus. HeLa cells, which are non-permissive for replication of E1-deleted adenovirus, were infected with 1-4 x 10^9 infectious units of recombinant adenovirus, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild type contamination, readily evident by the CPE observed in control cells infected with wild type adenovirus at a level of sensitivity of approximately 1 in 10^9.

p53 Protein Expression from Recombinant Adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines which do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma) were infected for 24 hours with the p53 recombinant adenoviruses A/M/53 or A/C/53 at MOIs ranging 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in non-infected cells. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek *et al*, 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower MOIs (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53 Dependent Morphology Changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a

characteristic enlargement and flattening of these normally spindle-shaped cells (Chen *et al*, 1990). Subconfluent Saos-2 cells ($1x10^5$ cells/10cm plate) were infected at an MOI of 50 with either the A/C/53 or control A/M virus, and incubated at 37ºC for 72 hours until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53 treated plate (Fig. 3, panel C) but not in uninfected (Fig. 3, Panel A) or control virus-infected plates (Fig. 3, Panel B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hours when its confluence approximated that of the A/C/53 treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at an MOI of 50 in Saos-2 cells (Fig.2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 Inhibition of Cellular DNA Synthesis

To further test the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of ^{3}H-thymidine. It has previously been shown that introduction of wild-type p53 into cells which do not express endogenous wild-type p53 can arrest the cells at the G_1/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker *et al*, 1990, Mercer *et al*, 1990, Diller *et al*, 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53 expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4) was observed. Both constructs were able to inhibit DNA synthesis in these human tumor

cells, regardless of whether they expressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at an MOI as low as 10. At doses where inhibition by the control adenovirus in only 10-30%, we observed a 50-100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53, Bressac *et al*, 1990), nor in the K562 (p53 null) leukemic cell line.

Tumorigenicity in Nude Mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells *ex vivo* and then injected the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a MOI of 3 or 30, were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8 week period. At the MOI of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Figure 5). The progressive enlargement of the control virus treated tumors were similar to that observed in the buffer treated control animals. A clear difference in tumor growth between the control adenovirus and the p53 recombinant at the MOI of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks. Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment.

In Vivo **Expression of rAd/p53**

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, $p53^{null}$) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2×10^9 pfu of either A/C/53 or A/C/ß-gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either Day 2 or Day 7 following the adenovirus injection, and polyA RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53 treated tumors (Fig. 6, lanes 1,2,4,5). No p53 signal was evident from the tumors excised from the ß-gal treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7-9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53 specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least one week following infection with a p53 recombinant adenovirus.

In Vivo **Efficacy**

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of

buffer or recombinant virus twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). After cessation of injections, the control treated tumors continued to grow while the p53 treated tumors showed little or no growth for at least one week in the absence of any additional supply of exogenous p53 (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus treated group, no significant difference in body weight was found between the three groups during the treatment period. Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantage for the p53-treated animals (Fig. 7B). The last of the control adenovirus treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 continue to survive (day 110) (Fig. 7B). Together, this data establish a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

Adenovirus Vectors Expressing p53

Recombinant human adenovirus vectors which are capable of expressing high levels of wild-type p53 protein in a dose dependent manner were constructed. Each vector contains deletions in the E1a and E1b regions which render the virus replication deficient (Challberg and Kelly, 1979, Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19 and 55 kd protein is able to bind wild-type p53 protein (Sarnow *et al*, 1982, Heuvel *et al*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function are removed through direct binding to p53 or potential inhibition of p53 mediated apoptosis. Additional constructs were constructed which have had the remaining 3′ E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb less than wild-type virus (Ghosh-Choudhury *et al*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 base pairs, decreasing the chances of regenerating replication-competent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy to those with pIX.

p53/Adenovirus Efficacy *In Vitro*

In concordance with a strong dose dependency for expression of p53 protein in infected cells, a dose-dependent, p53-specific inhibition of tumor cell growth was demonstrated. Cell division, was inhibited and demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53

protein expression. Bacchetti and Graham (1993) recently reported p53 specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, additional human tumor cell lines were demonstrated, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At MOIs where the A/C/N/53 recombinant is 90-100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus mediated suppression is less than 20%.

Although Feinstein *et al* (1992) reported that re-introduction of wild-type p53 could induce differentiation and increase the proportion of cells in G_1 versus $S+G_2$ for leukemic K562 cells, no p53 specific effect was found in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiment s, able to significantly infect the non-responding K562 cells with recombinant A/C/ß-gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable. Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of 4 p53 treated animals at the lower MOI most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. The complete suppression seen with A/M/N/53 at the higher dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus *In Vivo* Efficacy

Work presented here and by other groups (Chen *et al,* Takahashi et al, 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. Applicant presents the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53 expressing adenovirus treated tumors. However, both p53 and control virus treated tumor groups showed tumor suppression as compared to buffer treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon-*y*), interleukin (IL)-2, IL-4 or IL-7 can lead to T-cell independent transient tumor suppression in nude mice (Hoch *et al,* 1992). Exposure of monocytes to adenovirus virions are

also weak inducers of IFN-α/ß (reviewed in Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice was observed even with the control adenovirus. This virus mediated tumor suppression was not observed in the *ex vivo* control virus treated Saos-2 tumor cells described earlier. The p53-specific *in vivo* tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 110 days after their last injections compared to 0 out of 5 adenovirus control treated animals. The surviving animals still exhibit growing tumors which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer *et al*, 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment. For example, mutations in the recently described WAF1 gene, a gene induced by wild-type p53 which subsequently inhibits progression of the cell cycle into S phase, (El-Deiry *et al*, 1993, Hunter, 1993) could result in a p53-resistant tumor.

Implications for Gene Therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration associated with human cancers, occurring in 50-60% of human cancers (Hollstein *et al*, 1991, Bartek *et al*, 1991, Levine, 1993). The goal of gene therapy in treating p53 deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptisis

can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or treatment with some chemotherapeutic agents (Lowe *et al*, 1993A,B). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptisis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Towards that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1994). Replication deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromasomal DNA will be gradually lost with continued division of normal cells. Stable, high titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus mediated gene delivery has a strong potential for gene therapy for

diseases such as cystic fibrosis (Rosenfeld *et al*, 1992, Rich *et al*, 1993) and α_1-antitrypsin deficiency (Lemarchand *et al*, 1992). Although other alternatives for gene delivery, such as cationic liposome/DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus mediated gene delivery.

Here, recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA synthesis and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, recombinant adenoviruses can express p53 in an *in vivo* established tumor without relying in direct injection into the tumor or prior *ex vivo* treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth *in vivo* and significantly increased survival time in a nude mouse model of human lung cancer.

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Although the invention has been described with reference to the above embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims that follow.